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13. ABSTRACT ***(Maximum 200 Words)***

An early first full-term pregnancy significantly reduces a woman's lifetime risk of developing breast cancer. As such, early first childbirth is one of the most effective naturally occurring protective events that can diminish a woman's risk of breast cancer and is a candidate for targeted chemopreventive strategies. Despite extensive epidemiological evidence in support of parity-induced protection against breast cancer, very little is known about the molecules and pathways responsible for this protective effect. Rodent carcinogenesis models mimic the epidemiology of early parity and provide a valuable system for examining its biological underpinnings. As a means of addressing the underlying molecular and cellular basis of parity-induced protection, we have conducted a broad-based gene expression analysis of nulliparous and parous murine mammary glands. As a result of this analysis, we have generated a panel of genes that molecularly define the protected parous mammary gland, including differentiation markers, immune-related genes, growth factors and *TGF-β3*. We identified the upregulation of several differentiation markers for mammary epithelial cells in the parous mammary gland. Additionally, we isolated genes whose expression marks the presence of a permanent population of lymphocytes and macrophages residing in the parous mammary gland. Further analysis of major categories of genes whose expression correlates with the protected parous state revealed a downregulation of multiple growth promoting molecules, such as amphiregulin, insulin-like growth factor, and pleiotrophin, concomitant with an upregulation of growth-inhibitory signaling involving *TGF-β3* and *clusterin*. To date, these findings represent the most comprehensive analysis of molecular differences induced in the mammary gland by parity. In addition, these data provide molecular evidence for mechanisms previously proposed for parity-induced protection as well as providing novel insight into additional pathways involved in this process.

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Celina M. DCrs 10/25/00

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Introduction:

An early first full-term pregnancy significantly reduces a woman's lifetime risk of developing breast cancer. As such, early first childbirth is one of the most effective naturally occurring protective events that can diminish a woman's risk of breast cancer and is a candidate for targeted chemopreventive strategies. Despite extensive epidemiological evidence in support of parity-induced protection against breast cancer, very little is known about the molecules and pathways responsible for this protective effect. Rodent carcinogenesis models mimic the epidemiology of early parity and provide a valuable system for examining its biological underpinnings. As a means of addressing the underlying molecular and cellular basis of parity-induced protection, we have conducted a broad-based gene expression analysis of nulliparous and parous murine mammary glands. As a result of this analysis, we have generated a panel of genes that molecularly define the protected parous mammary gland, including differentiation markers, immune-related genes, growth factors and *TGF-β3*. We identified the upregulation of several differentiation markers for mammary epithelial cells in the parous mammary gland. Additionally, we isolated genes whose expression marks the presence of a permanent population of lymphocytes and macrophages residing in the parous mammary gland. Further analysis of major categories of genes whose expression correlates with the protected parous state revealed a downregulation of multiple growth promoting molecules, such as amphiregulin, insulin-like growth factor, and pleiotrophin, concomitant with an upregulation of growth-inhibitory signaling involving *TGF-β3* and *clusterin*. To date, these findings represent the most comprehensive analysis of molecular differences induced in the mammary gland by parity. In addition, these data provide molecular evidence for mechanisms previously proposed for parity-induced protection as well as providing novel insight into additional pathways involved in this process.

BODY

SPECIFIC AIMS:

Aim I. Identify molecular markers demonstrating parity-related changes in the rat breast.

Isolate candidate markers whose expression correlates with the protected state of the parous mammary gland using screening methods to identify differentially expressed cDNA.

Aim II. Characterize temporal and spatial expression of molecular markers in the mammary gland.

Confirm the temporal and spatial pattern of molecular marker expression in the mammary gland as a result of parity to determine the mechanism of differential regulation of candidate cDNA markers.

Aim III. Determine the expression of molecular markers with respect to parity-related reproductive histories.

Generate mammary gland tissue from animals with varying reproductive histories in order to determine parity-dependent expression of the candidate genes isolated in Specific Aim 1 and confirmed in Specific Aim 2.

TECHNICAL OBJECTIVES:

Task 1. To identify molecular markers demonstrating parity-related changes in rat breast tissue: months 1-24

- mate animals for virgin and parous breast tissue, harvest and purify total RNA: months 1-6
- generate cDNA libraries from virgin and parous rat breast tissue: months 6-12
- initiate differential screening of cDNA libraries: months 6-12
- initiate PCR-subtractive hybridization: months 6-12
- purify, sequence, and confirm expression of candidate markers: months 8-24

Mate virgin and parous animals to harvest and purify total RNA. (months 1-24)

In order to increase the number of differentially expressed genes identified between virgin and parous mammary glands, we have investigated the availability of murine microarray technologies. Since this technology has proven to be very effective in distinguishing differentially expressed cDNA in a variety of other tissues, we have also employed this technology to increase our pool of molecular markers from the panel of genes identified through PCR-Select Subtractive Hybridization. In order to generate the starting tissue to use for microarray

technology, we have created 3 independent pools of virgin and parous animals, consisting of 15-20 animals/pool. Parous animals were harvested following a single-round of pregnancy, lactation and 4 weeks of post-lactational regression along with age-matched littermates. Total RNA was prepared by homogenizing snap frozen tissue in gaunidine isothiocyanate and purified by ultracentrifugation through a CsCl gradient.

Screening Expression of Virgin and Parous Tissue by Oligonucleotide Microarrays. (months 12-18)

* in addition to proposed PCR-based techniques already employed (months 1-12)

We prepared cDNA and biotinylated complementary RNA from each of the 3 independent virgin and parous samples. Using hybridization cocktails from these samples, 11,000 genes and EST's were monitored for expression in virgin and parous mammary gland samples performed in triplicate. Using the Affymetrix software we were then able to generate a list of genes whose differential expression are highly reproducible (see manuscript, Fig. 2, Table 1). The results of our microarray analysis confirmed all of the candidate genes isolated by PCR-based subtractive hybridization and cDNA library screening techniques.

Task 2. To characterize the temporal and spatial expression of candidate molecular markers: months 12-36

- generate parity-related developmental timepoints, prepare RNA and histological sections: months 12-18
- test temporal expression pattern of candidate markers: months 12-36
- perform *in situ* hybridization of interesting candidate markers: months 12-36

Generate parity-related developmental time points, prepare RNA and histological sections: months 12-18

In order to address the mechanism by which the expression of differentially expressed cDNA are altered as a result of parity, we generated cohorts of animals and mammary gland tissue at a variety of reproductive time points relevant to parity. These points include virgin development (2,5,10 and 15 weeks), pregnancy (D6, D12, and D18), lactation (D9), and post-lactation involution (D2, D4 and D28). Mammary gland tissue were harvested from animals at these discrete developmental milestones and processed for both mRNA and paraffin-embedded blocks.

Test temporal expression pattern of candidate markers: months 12-36

In order to demonstrate the temporal pattern of expression of many of the differentially expressed cDNA isolated by oligonucleotide microarrays, we monitored expression of our cDNA markers by Northern analysis throughout mammary gland development (see manuscript, Fig. 3, 4 and 5). This analysis

demonstrated distinct patterns of expression by which the expression of molecular markers are altered by parity, including a down-regulation during pregnancy, and up-regulation of expression during pregnancy, lactation and involution.

Perform *in situ* hybridization of interesting candidate markers: months 12-36

To determine the spatial pattern of expression of molecular markers identified by microarray analysis, we performed *in situ* hybridization on sections from different developmental time points. By this analysis, we were able to distinguish between cDNA that mark mammary epithelial cells, as well as adipocytes, lymphocytes and macrophages (see manuscript, Fig. 3,4 and 5, data not shown).

Task 3. To generate animals of various reproductive histories and test candidate markers for their parity-dependence: months 12-36

- generate animals with the required reproductive history, prepare RNA, and histological sections: months 12-36.
- test temporal expression pattern of candidate markers: months 12-36
- perform *in situ* hybridization of interesting candidate markers: 12-36

Generate animals with the required reproductive history, prepare RNA, and histological sections: months 12-36.

As a means of determining whether the expression of the panel of molecular markers are permanent during longer periods or regression, we have generated cohorts of animals that have had longer periods of post-lactational involution, such as 16 and 30 weeks, in addition to 4 weeks of regression. We have also generated groups of animals with varying ages of mating and their respective age-matched littermates. Mammary gland tissue was prepared for mRNA and paraffin-embedded blocks. Expression of candidate genes have been tested to by Northern analysis and have confirmed for a subset of genes that their parity-dependent pattern is insensitive to length of regression (see manuscript, Fig. 6 and 7).

KEY RESEARCH ACCOMPLISHMENTS

- Generated a panel of molecular markers that are differentially expressed between nulliparous and parous murine mammary glands.

- Provided the first molecular evidence that parity results in a more differentiated mammary gland.

- Identified genes that demonstrate that the there is an increase in immune-related cells in the parous mammary gland as compared to the virgin mammary gland.

- Identified a number of growth factors whose expression decreases as a function of parity.

- Demonstrated that cell growth inhibitory pathways such as TGF-B3 and its downstream targets are up-regulated as a result of parity.

- Provided evidence that the markers identified in this study are highly conserved and permanent throughout regression.

REPORTABLE OUTCOMES

Publication of manuscripts supported by this grant:

Chodosh, LA, D'Cruz, CM, Gardner, HP, Ha, SI, Marquis, ST, Rajan, JV, Stairs, DB, Wang, JY, Wang, M. Mammary gland development, reproductive history, and breast cancer risk. Cancer Res (1999) 59(7 Suppl):1765-1771s;discussion

D'Cruz, C.M., Moody, S.E., Master, S.R., Hartman, J. L., Cox, J.D., Wang, J.Y., Ha, S.I., Stoddard, A., and Chodosh, L.A. Early Pariy Results in Permanent Changes in Gene Expression in the Murine Mammary Gland. (Manuscript in preparation).

D'Cruz, CM, Ha, SI, Wang, JY, Marquis, ST, and Chodosh, LA. Parity-Induced Differentiation is Dependent on Reproductive History. (Manuscript in preparation).

CONCLUSIONS

By microarray technology, we have identified a panel of cDNA whose expression correlates with the protected state following an early first full-term pregnancy. Using this analysis, we have been able to detect both gross and subtle changes in gene expression that reproducibly distinguish between nulliparous and parous glands, as well as to identify changes in cell types that occur as a result of early parity. We have found molecular evidence that parity results in a more differentiated mammary epithelium, and that there is an increased contribution of the immune system in the parous gland. In addition, we have identified changes in regulatory pathways that have the potential to alter the susceptibility of the mammary epithelial cells to malignant transformation. Finally, the expression of several of these markers appears to be insensitive to length of regression following a pregnancy, suggesting that the differential patterns that we have identified are permanent. Moreover, the parity-specific pattern of expression of many of the genes discussed in this study have been demonstrated in the rat, and indicate that many of the molecular changes that we have defined are highly conserved and may be relevant to the human breast.

REFERENCES

See appendices (page 40-51)

Early Parity Results in Permanent Changes in Gene Expression in the Murine Mammary Gland

Celina M. D Cruz *, Susan E. Moody *, Stephen R. Master, Jennifer Hartman, James D. Cox, James Y. Wang, Seung I. Ha, Alex Stoddard and Lewis A. Chodosh

ABSTRACT

An early first full-term pregnancy significantly reduces a woman's lifetime risk of developing breast cancer. As such, early first childbirth is one of the most effective naturally occurring protective events that can diminish a woman's risk of breast cancer and is a candidate for targeted chemopreventive strategies. Despite extensive epidemiological evidence in support of parity-induced protection against breast cancer, very little is known about the molecules and pathways responsible for this protective effect. Rodent carcinogenesis models mimic the epidemiology of early parity and provide a valuable system for examining its biological underpinnings. As a means of addressing the underlying molecular and cellular basis of parity-induced protection, we have conducted a broad-based gene expression analysis of nulliparous and parous murine mammary glands. As a result of this analysis, we have generated a panel of genes that molecularly define the protected parous mammary gland, including differentiation markers, immune-related genes, growth factors and *TGF-β3*. We identified the upregulation of several differentiation markers for mammary epithelial cells in the parous mammary gland. Additionally, we isolated genes whose expression marks the presence of a permanent population of lymphocytes and macrophages residing in the parous mammary gland. Further analysis of major categories of genes whose expression correlates with the protected parous state revealed a downregulation of multiple growth promoting molecules, such as amphiregulin, insulin-like growth factor, and pleiotrophin, concomitant with an upregulation of growth-inhibitory signaling involving *TGF-β3* and *clusterin*. To date, these findings represent the most comprehensive analysis of molecular differences induced in the mammary gland by parity. In addition, these

data provide molecular evidence for mechanisms previously proposed for parity-induced protection as well as providing novel insight into additional pathways involved in this process.

MATERIALS AND METHODS

Animals and Tissues

FVB mice and Sprague-Dawley rats were housed under barrier conditions with a 12-h light/dark cycle and access to food and water *ad libitum*. Parous rodents were generated by mating 4-week-old mice or 9-week-old rats. Following parturition, the animals were allowed to lactate for 21 days, at which time their pups were weaned. They then underwent 4 weeks of post-lactational involution. At this time the #3-5 mammary glands were harvested from parous and age-matched nulliparous animals and snap frozen. With the exception of glands used for whole mounts, lymph nodes within gland #4 were visualized and removed.

Whole Mounts and Histology

Number 4 mammary glands were mounted on glass slides, fixed overnight in neutral buffered formalin, and transferred to 70% ethanol. For whole mounts, glands were rinsed in water for 5 min and stained in a filtered solution of 0.2% carmine (Sigma) and 0.5% aluminum potassium sulfate for 1-3 days. They were then transferred sequentially into 70% ethanol, 90% ethanol, and 100% ethanol for 15 minutes per wash and were de-fatted and stored in methyl salicylate. For histological analysis, fixed glands were blocked in paraffin, sectioned, and stained with hematoxylin and eosin.

RNA Isolation and Northern Analysis

Snap-frozen tissue was homogenized in guanidine thiocyanate supplemented with 7μl/ml 2-mercaptoethanol, and RNA was isolated by centrifugation through cesium chloride as

previously described. RNA was quantified and equal amounts from 15-20 mice or 10 rats were combined for each independent pool. Total RNA was separated on a 1% LE agarose gel, and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as per manufacturer s instructions using PerfectHyb Plus Hybridization Buffer (Sigma) and ^{32}P-labeled cDNA probes corresponding to Genbank sequences represented on the Affymetrix Mu11K Gene Chip.

Oligonucleotide Microarray Hybridization and Analysis

Approximately 40 μg of total pooled RNA from each sample was used to generate biotinylated cRNA as described(Lockhart et al., 1996). Hybridization to a set of two Affymetrix Mu11K GeneChips was performed as per manufacturers instructions. After washing and staining, chips were scanned using a Hewlett-Packard Gene Array Scanner. Grid alignment and raw data generation was performed using Affymetrix GeneChip 3.1 software.

***In Situ* Hybridization**

In situ hybridization was performed as described (Marquis et al., 1995). Antisense and sense probes were sythesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing the sequences used for Northern Hybridization analysis.

INTRODUCTION

Epidemiological data have consistently demonstrated that a woman s lifetime risk of developing breast cancer is significantly reduced if she undergoes her first full-term pregnancy at an early age. This protective effect is most profound when pregnancy occurs before the age of 20 and provides a greater than 50% reduction in long-term breast cancer risk as compared to women who never bear a child (Bain et al., 1981; Brinton et al., ; Carter et al., 1989; Ewertz et al., 1990; Helmrich et al., 1983; Layde et al., 1989; Leon, 1989; Lubin et al., 1982; Lund, 1991; MacMahon et al., 1970; MacMahon et al., 1982; Negri et al., 1988; Paffenbarger et al., 1980; Rosner and Colditz, 1996; Rosner et al., 1994; Salber et al., 1969; Tulinius et al., 1978; Wang et al., 1992; Yuan et al., 1988). The level of protection conferred by parity decreases with increasing age at first childbirth. In fact, women who first give birth after the age of 30 appear to incur a slightly higher lifetime risk of breast cancer than do nulliparous women. These observations suggest that reproductive events occurring early in a woman s lifetime have a significant impact on her risk of developing breast cancer many years later, and that the timing of such events is critical for determining this risk. In addition, early parity has been consistently demonstrated to provide a protective effect in women from diverse ethnic and racial groups as well as from varied geographic locations (Bain et al., 1981; Brinton et al., ; Carter et al., 1989; Ewertz et al., 1990; Helmrich et al., 1983; Layde et al., 1989; Leon, 1989; Lubin et al., 1982; Lund, 1991; MacMahon et al., 1970; MacMahon et al., 1982; Negri et al., 1988; Paffenbarger et al., 1980; Rosner and Colditz, 1996; Rosner et al., 1994; Salber et al., 1969; Tulinius et al., 1978; Wang et al., 1992; Yuan et al., 1988). The universality of this phenomenon suggests that the

protective effects of early first childbirth are intrinsic to the breast and are not solely due to environmental or genetic factors. Although other parity-related reproductive events, such as multiparity, spacing of pregnancies, and lactation history have also been reported to influence breast cancer risk, these effects are modest in comparison to that of age at first full-term pregnancy (Bruzzi et al., 1988; Kvale and Heuch, 1987; Kvale et al., 1987; Layde et al., 1989; Paffenbarger et al., 1980; Pathak and Whittemore, 1992; Vatten and Kvinnsland, 1992; Wang et al., 1992; Yuan et al., 1988). As such, parity-induced protection is one of the most effective naturally occurring events that reduces a woman s lifetime risk of developing breast cancer.

The biological basis for parity-induced protection has yet to be elucidated. Multiple events that take place during and after pregnancy, both systemically and within the mammary gland, may synergize to result in a more protected epithelium. This protected state could reflect pregnancy-driven terminal differentiation of target cells within the epithelium, as well as the preferential loss of target cells during the wave of apoptosis that accompanies post-lactational involution. Additionally, permanent changes in circulating hormone levels resulting from pregnancy may modulate the mammary gland s susceptibility to tumor formation. Consistent with a model in which both extrinsic and intrinsic changes contribute to parity-induced protection against breast cancer is the observation that parity reduces circulating levels of prolactin and growth hormone and also results in decreased levels of epidermal growth factor and estrogen receptor expression in the parous mammary epithelium (Musey et al., ; Thordarson et al., 1995).

Similar to epidemiologic findings in humans, the protective effect of parity has also been demonstrated in rodent models. Both rats and mice that have undergone an early full-term

pregnancy are less susceptible to carcinogen-induced tumorigenesis than nulliparous animals (Dao et al., 1960; Huggins et al., 1959; Huggins et al., 1959; Medina and Smith, 1999; Moon, 1981; Moon, 1969; Russo and Russo, 1978; Russo et al., 1977; Russo et al., 1979). The contribution of specific ovarian and placental hormones to the protective effect of pregnancy has also been studied in the rat model. In such studies, short-term treatment of nulliparous rats with either 17-β estradiol plus progesterone or with human chorionic gonadotropin has been demonstrated to provide protection against mammary tumorigenesis to an extent equivalent to that observed in parous animals (Guzman et al., 1999; Russo et al., 1990; Russo et al., 1990). While it is not feasible to achieve the levels of hormones used in these studies in humans, these results suggest that the protective effect of parity may be able to be mimicked pharmacologically. As such, elucidation of the molecular pathways responsible for parity-induced protection against breast cancer will likely provide a more targeted strategy for defining a protected state in the mammary gland, and should facilitate the development of feasible chemopreventative regimens.

Despite long-standing evidence for the differential susceptibility of the parous and nulliparous breast to carcinogenesis, to date there has been no comprehensive description of the molecular and cellular differences between the parous and nulliparous mammary gland. Such data would not only provide a means by which to molecularly define a protected state of the mammary gland, but would also provide insight into pathways that are potentially responsible for parity-induced protection. Moreover, defining a panel of molecular markers whose expression is highly correlated with early parity, irrespective of their function, should provide a means to monitor the efficacy of pharmacological interventions designed to mimic this naturally occurring

protected state.

In this report, we have used microarray technology to conduct a broad-based gene expression analysis of mammary glands from age-matched nulliparous and parous mice. Through this study we have identified a panel of markers whose expression in the mammary gland changes as a consequence of parity. Analysis of such genes indicates that parity results in a more differentiated mammary gland. In addition, the upregulation of genes specific to the immune system suggests that parity permanently alters the cellular composition of the mammary gland. Further analysis of genes differentially expressed in the parous mammary gland also reveals the downregulation of several growth-promoting molecules, whereas a growth-inhibitory pathway appears to be upregulated. In addition to providing molecular evidence for previously proposed mechanisms of parity-induced protection, our results suggest novel pathways whose regulation may contribute to the protected state of the parous mammary gland.

RESULTS

Parity Results in Permanent Morphological Changes in the Mammary Gland

Parity-induced changes in breast cancer susceptibility are paralleled in humans by morphological alterations in the mammary gland (Daniel and Silberstein, 1987; Russo et al., 1990). These structural changes are permanent and suggest a more differentiated morphological state of the parous breast as compared to the nulliparous breast (Russo et al., 1992; Russo and Russo, 1993). Notably, morphological differences between nulliparous and parous mammary glands have also been observed in rodent models (Russo et al., 1982). To extend our understanding of the structural changes that occur as a result of parity to additional mouse

systems, we compared the morphology of mammary glands from nulliparous and parous rats with those of two independent mouse strains. Parous animals in this study underwent a single round of pregnancy, lactation, and 28 days of post-lactational involution. FVB and C57/Bl6 mice were mated at 4-6 weeks of age, and Sprague-Dawley rats were mated at 8-9 weeks of age. These time periods correspond to the onset of puberty in these animals and thus represent early parity. Carmine-stained whole mounts of nulliparous and parous mammary glands demonstrate that the epithelial tree in the parous gland is more highly branched than in the age-matched nulliparous gland (Fig. 1). These marked differences in the structure of the parous and nulliparous ductal epithelial tree are similar between rats and different mouse strains examined (Fig. 1). Moreover, these differences persist even after 30 weeks of regression (data not shown), .indicating that the morphological changes induced by parity are essentially permanent. Despite the gross differences in ductal branching between nulliparous and parous mammary glands, hematoxylin and eosin-stained cross-sections demonstrate that the relative amount of epithelium embedded within the mammary fat pad is approximately equivalent at both development stages (Fig. 1). These consistent and easily distinguishable architectural differences between nulliparous and parous rodent glands are analogous to those that have been described in the human breast (Russo et al., 1990; Russo et al., 1992).

Microarray Analysis Identifies Parity-Induced Changes in Gene Expression

Given the evidence that both structural and functional parity-induced changes are conserved from humans to rodents, we sought to define the molecular differences between nulliparous and parous mammary glands in a murine system. In order to characterize these

differences as comprehensively as possible, we employed microarray technology to monitor the expression profiles of 11,000 genes and EST's. Although mammary adenocarcinomas arise from epithelial cells, we believed it to be essential to analyze the entire mammary gland as other cellular compartments have been clearly shown to affect the signaling and growth of mammary epithelial cells (Wiesen et al., 1999). By performing a broad-based comparison between two developmental states with known differences in cancer susceptibility, we hoped to generate a robust panel of molecular markers whose expression patterns define this protected state. We hypothesized that such a panel of molecular markers would also facilitate the identification of cell types within the mammary gland, thereby allowing us to monitor changes in their relative distribution as a consequence of parity. We anticipated that the majority of differentially expressed genes identified by this approach would serve to define the protected state of the mammary gland at the cellular and molecular level, but would not be expected to play a causative role in parity-induced protection. However, we also believed that a subset of these genes would likely provide insight into the functional alterations in the parous gland that are ultimately responsible for changes in the susceptibility to tumorigenesis.

Each sample in this analysis consisted of pooled mammary gland total RNA which was derived from 15-20 animals in order to control for animal-to-animal fluctuations in gene expression levels that occur as a result of the estrus cycle and other sources of biological variability (Robinson et al., 1995). To maximize the reproducibility of our results, three independent, age-matched pools for nulliparous (15 wk G0P0) and parous (15 wk G1P1) cohorts were generated. Using standard methods, cDNA was generated from each pool of total RNA and reverse transcribed to prepare target cRNA for the hybridization reactions. Samples were

hybridized to oligonucleotide microarrays representing approximately 11,000 murine genes and ESTs. Raw gene expression levels were scaled to all genes, and these normalized data sets were compared to each other to generate lists of candidate markers whose expression levels consistently changed as a function of parity.

Our first level of analysis involved the confirmation by Northern of all genes that were identified as differentially expressed by Affymetrix algorithms in the three independent microarray experiments. cDNA fragments corresponding to the specific Genbank sequences as identified on the oliognucleotide microarray were cloned by RT-PCR, radiolabeled and hybridized to Northern membranes containing total RNA from three independent pools of nulliparous and parous mammary glands. One of these pairs of pooled samples had also been subjected to microarray analysis (Fig. 2, lanes 1 and 4), while the other two represented additional independent nulliparous and parous pools (Fig. 2, lanes 2, 3, 5, and 6). We found that, when restricting our analysis to sequences reproduced on the microarrays in triplicate, our true positive rate as confirmed by Northern was 100% (14/14). Given this high true positive rate, we expanded our analysis to include genes that were identified as differentially expressed in two out of three experiments, with a minimum fold change of 1.5 in the third experiment. The majority of these genes were also confirmed by Northern analysis (Table 1).

We further reasoned that the detection of relatively small fold changes in gene expression was of high importance, as small changes in the expression of regulatory molecules or in the abundance of certain cell types could have a significant impact on overall cellular function and differentiation status. By our method of microarray analysis we were able to identify and confirm genes whose difference in expression between the nulliparous and parous state was as

subtle as 1.7- fold. As evidenced by Northern analysis of the nulliparous-specific genes *superoxide dismutase III (SOD 3), Ob*, and *MUC18*, and of the parous-specific genes *carbonic anhydrase isozyme II (CAII), adipocyte differentiation-related protein* (*ADFP*) and *adenosine deaminase* (*ADA*), even small fold changes are highly consistent across independent pools of animals (Fig. 2). These data imply that these genes represent true biological markers that change as a consequence of parity. As expected, our analysis was also able to identify markers that consistently display relatively large changes in expression levels, such as a homologue to the Ig Superfamily and *carboxyl ester lipase* (CEL) (Fig. 2). Relatively constant levels of expression of an epithelial marker, *cytokeratin18*, in nulliparous and parous mammary glands confirmed that the detected changes were not merely a consequence of expansion or contraction of the epithelial cell compartment (Fig. 2). From these data it is clear that the molecular definition of differences between the nulliparous and parous gland includes both gross and subtle changes in gene expression.

Differentiation Markers are Preferentially Expressed in the Parous Mammary Gland

From our panel of molecular markers, we identified several distinct functional categories of genes whose expression was altered as a result of parity (Table 1). A prominent category of markers whose expression increased in the parous mammary gland included markers for mammary epithelial differentiation, such as the milk protein genes, *α-casein, β-casein, γ-casein, κ-casein, whey acidic protein, and alpha-lactalbumin* (Table 1). We also identified genes with reported functions in milk production, such as *butyrophilin,* and *GLYCAM-1*, that may also represent markers for terminally differentiated epithelial cells (Table 1) (Mather and Jack, 1993;

Nishimura et al., 1993; Ogg et al., 1996). Similarly, the parous-specific expression of *connexin 26* and *30* may reflect the more differentiated state of the parous mammary gland, as members of the connexin family are thought to be essential for gap junction formation in secretory alveolar epithelial cells (Locke et al., 2000). Of note, the finding that *ADFP*, a stromal-specific marker that is reported to be transcriptionally upregulated in differentiated adipocytes, is more highly expressed in the parous gland suggests that stromal compartments as well as epithelial compartments of the parous mammary gland may be more differentiated (Gao et al., 2000). Thus, our findings provide the first molecular evidence supporting the hypothesis that parity results in a more differentiated mammary gland (Russo et al., 1982).

Parity-Induced Changes in Immune-Related Cells in the Mammary Gland

Further analysis of the parous-specific markers identified above revealed a subset of genes including *kappa light chain*, and the heavy chains of *IgG, IgM, IgA*, as well as *macrophage metalloelastase (MME)* and *early T-lymphocyte activation protein (Eta-1)* which are generally expressed by cells of the immune system (Table 1). The differential expression of these markers implies that lymphocytes and macrophages may be present in more abundant numbers in the parous as compared to the nulliparous mammary gland. If this is true, our data suggest that parity results in changes in cellular populations within the parous breast. To address the contribution of immune-related cells to parity-induced changes in the mammary gland, we initiated a study of representative genes for the different cell types identified by our screen. Since κ-LC forms a complex with the immunoglobulin heavy chain genes γ, μ and α, we utilized this gene as a marker for B-lymphocytes. In addition, we focused on *MME* and *Eta-1* as genes

that may mark selected immune cells. Although *MME* expression has been reported in stromal cells and T-lymphocytes, it is primarily expressed by macrophages (Belaaouaj et al., 1995). Similarly, *Eta-1* expression has been reported in non-immune cells, such as mammary epithelial cells as well as immune-related cells, including macrophages and T-lymphocytes (Ashkar et al., 2000; Tuck et al., 1999).

To explore the contribution of immune cells in the parous breast, we initially hybridized a probe for *MME* to three independent pools of total RNA from nulliparous and parous mice (Fig. 3A). Steady state mRNA levels for *MME* were consistently elevated in parous samples over levels seen in nulliparous samples (Fig. 3A). In order to address the temporal regulation of *MME* and the mechanism for parity-induced expression, we monitored expression of *MME* during several stages representative of postnatal mammary gland development. These points included male mammary gland (15 wk) and female mammary glands corresponding to preadolescence (2 wk), puberty (5 wk), adult (10 and 15wk), pregnancy (Day 6, Day 12, Day 18), lactation (Day 9) and regression (Day 2, Day 7, Day 28). Both Northern and *in situ* hybridization analyses demonstrate that MME expression during mammary gland development dramatically increases during mid regression (Day 7) with levels of expression remaining high during late-regression (Day 28)(Fig. 3B). This temporal pattern of expression is consistent with previous evidence that macrophages are recruited to the breast in order to clear post-apoptotic debris during involution (Belaaouaj et al., 1995; Strange et al., 1992). In addition, *in situ* analysis demonstrated that cells marked by *MME* expression were non-epithelial. While our analysis cannot distinguish whether the cell type marked by *MME* expression is of macrophage or T-cell lineage, the timing of expression for *MME* during mammary gland development suggests that *MME* is a marker for

macrophages in the breast.

We initially verified the parity-specific expression patterns of *κ-LC* by Northern analysis of nulliparous and parous mammary glands (Fig. 3A). In order to define the timing of the influx of B-lymphocytes to the breast, we monitored expression of *k-LC* during several major stages of mammary gland development. Northern analysis revealed that *κ-LC* expression levels were first detected in lactation (D9) with elevated levels demonstrated throughout regression (Fig. 3B). The increased levels of *κ-LC* expression during lactation are difficult to visualize due to dilutional effects that result from large-scale milk protein gene expression that also occur during this period (Marquis et al., 1995; Rajan et al., 1997). This dilutional effect is also apparent by monitoring the expression of β-actin over this same time period (Fig. 3B). The temporal pattern of expression *for κ-LC* is consistent with the reported influx of lymphocyte to the breast that occurs during lactation (Asai et al., 2000; Tanneau et al., 1999; Tatarczuch et al., 2000).

We performed *in situ* hybridization to determine whether the increase in *κ-LC* expression was in fact due to an influx of B-lymphocytes, and to address the location of B-lymphocytes with respect to the stromal and epithelial compartments of the mammary gland. This analysis confirmed the temporal pattern of *κ-LC* expression as demonstrated by Northern analysis (Fig. 3C). The spatial pattern of expression also demonstrated that B-lymphocytes are located in close proximity to the epithelium and are evenly distributed throughout the gland (data not shown).

To gain insight into the function of *Eta-1* in the mammary gland, we initially confirmed the parous-specific expression of this gene in the nulliparous and parous mammary gland (Fig. 3A). To elucidate the developmental pattern of regulation for *Eta-1* and the cell type contributing to this expression, we examined *Eta-1* expression during postnatal mammary gland development. *Eta-1* expression was first detectable during mid-pregnancy and exhibited maximal expression during late pregnancy (Fig. 3B). The apparent reduction in *Eta-1* expression that is observed during lactation is most likely due to the dilutional effects described above. *Eta-1* expression was elevated during early regression (Day 2, Day 7) and declined somewhat by Day

28 of regression but remained elevated relative to age-matched nulliparous animals (Fig. 3B). Interestingly, *in situ* analysis demonstrated that *Eta-1* expression was found predominantly in the mammary epithelium, although macrophage and T-lymphocyte expression can not be excluded. In aggregate, our data suggest that the parous mammary gland has an expanded population of macrophages, activated B-lymphocytes, and epithelial cells expressing *Eta-1*. At present, the implications of an increase in both cytokine expression and immune cells in the parous breast remain unclear. We speculate, however, that the composition of this cellular compartment may generate paracrine signaling between cells of the immune system and epithelial cells that could ultimately lead to reduced tumorigenicity in the parous mammary gland.

Parity Results in a Decrease in Growth Factor Expression

Interestingly, the number of genes downregulated as a result of parity was far less extensive than that of genes upregulated. The majority of these genes, however, are regulatory molecules and effectors of distinct signaling pathways within the cell. Genes in this category included *amphiregulin (AR), pleiotrophin (PTN), pre proinsulin-like growth factor* 1 *(IGF-1), ob (Ob), thyroid stimulating hormone receptor (TSHR)* and an EST homologous to the growth hormone receptor (Table 1). The hypothesis that hormone and growth factor receptor levels are decreased as a result of parity has been previously proposed (Thordarson et al., 1995). In support of this model, we have isolated additional molecules involved in mitogenic signaling pathways in the cell that appear to have reduced expression profiles in the parous gland. To verify the specific regulation of growth factors within the mammary gland, we investigated the expression of this class of genes in triplicate samples of nulliparous and parous animals. A

consistent downregulation in the expression of *AR, PTN, TSHR, IGF-1A* and *Ob* was detected in parous mammary glands as compared to nulliparous mammary glands for each gene (Fig. 4A and Fig. 2).

Although it is possible that a variety of mechanism for the downregulation of mitogenic signaling pathways may occur as a result of parity, we investigated the expression pattern of *AR,* an epidermal growth factor family member, during the course of development as a representative gene from this family of regulatory molecules. We hybridized a cDNA probe for *AR* to Northern membranes containing tissue from timepoints during distinct stages of mammary gland development. Expression of *AR* was first detectable at 5 wks in the female murine mammary gland. This period corresponds to a stage at which the mammary gland is undergoing ductal morphogenesis and rapid epithelial proliferation. Notably, the levels of *AR* remain constant throughout nulliparous development, even during times at which ductal elongation is essentially complete. Expression of *AR* appeared to decrease after the highly proliferative stage of pregnancy (Day 6) and remained low in the parous gland (Fig. 4B). By *in situ* analysis, we confirmed that expression of *AR* is globally downregulated in the parous epithelium (Fig. 4C). As such, the regulation of *AR* suggests that expression is consistent throughout nulliparous development and is maintained during the proliferative stages of early pregnancy. The downregulation of *AR* mRNA levels occurs during the late stages of pregnancy periods of decreased growth and increased differentiation in the mammary gland. Further analysis of the temporal regulation of growth factors is necessary to determine whether additional patterns of expression can contribute to a decrease in parous expression. Nonetheless, these findings extend

the molecular evidence that supports a model in which growth stimulatory pathways are downregulated as a consequence of parity.

Parity Results in Increased TGB-β3 Signaling in the Parous Breast

A subset of regulatory genes also demonstrated patterns of increased expression as a result of parity. We identified parity-induced upregulation of the transcription factors, *A-myb, OBF-1,* and *Id-2,* as well as the pluripotent cytokine *TGF-β3* (Table 1). While it is likely that the activation of cellular signaling pathways involves modification of relevant molecules at the protein level, we hypothesized that activation of some pathways will also lead to changes in transcription of target molecules. In support of this hypothesis is the coordinate upregulation of *TGF-β3, Id-2,* and *clusterin* (Table 1). *Id-2* has been implicated as a downstream transcriptional target induced by *TGF-β3* during granulocyte differentiation while *clusterin* is a known downstream target of *TGF-β3* that plays a role in apoptotic signaling (Table 1) (Cooper and Newburger, 1998; French et al., 1996; Jin and Howe, 1999). The identification of coordinate regulation of both a ligand and its downstream targets suggests that our analysis of gene expression patterns can provide insight into the regulation of molecular signaling pathways.

We initially confirmed the expression of *TGF-β3* and clusterin by Northern and *in situ* analysis. Probes complementary to *TGF-β3* and *clusterin* sequences were hybridized to replicate Northern blots containing three independent nulliparous and parous total RNA samples. Both *TGF-β3* and *clusterin* were expressed at relatively low levels within the mammary gland, consistent with levels detected for other regulatory molecules in the breast (Fig. 5A, data not shown). Parous expression of *TGF-β3* and *clusterin* was uniformly elevated over nulliparous

expression (Fig. 5A). This result suggests that parity has permanently altered either the level of expression of these molecules within a cell or the relative abundance of cells expressing these markers.

We investigated the temporal expression of *TGF-β3* and *clusterin* during mammary gland development in order to address the timing in which expression increased as a result of parity. *TGF-β3* and *clusterin* exhibited maximal expression at Day 2 of involution and decreased thereafter though remaining elevated (Fig. 5B). Day 2 of post-lactational involution corresponds to the period when the mammary gland is undergoing a dramatic wave of apoptosis. The similar expression profiles of both *TGF-β3* and *clusterin*, suggest that these molecules contribute to cell death within the mammary gland, a hypothesis that is consistent with previous studies in the breast (French et al., 1996; Nguyen and Pollard, 2000). In addition, the identical temporal patterns of expression as seen for *TGF-β3* and *clusterin* strongly suggest that these molecules are co-regulated, or that they are involved in similar pathways. The parallel expression profiles over mammary gland development are confirmed by in situ analysis as well (Fig. 5C). The increased expression pattern of *TGF-β3* and downstream targets is highly suggestive of activation of this pathway in the parous mammary gland and may provide a mechanism for increased growth-inhibitory signaling in the more protected parous state.

Parity-Dependent Expression of Genes is Independent of Reproductive Variables

We have isolated a panel of genes whose expression correlates with reproductive history and breast cancer risk. Although our data do not provide proof that the molecules and pathways described in this study are mechanistically involved in parity-induced protection, we hypothesize

that the expression profiles of this panel of genes correlates with reduced susceptibility to mammary cancer. If this hypothesis is true, we postulate that the differential regulation of such molecules will persist during longer period of regression, as parity-induced protection following an early first full-term pregnancy is thought to provide long-term protection. In order to address this concern, we generated cohorts of mice that were mated at 4 weeks of age, followed by 21 days of lactation and either 4, 16, or 30 weeks of post-lactational involution. Age-matched nulliparous animals were used as controls. Expression of proposed regulatory molecules such as *TGF-β3*, *clusterin*, and *Eta-1* were consistently upregulated in the parous mammary gland irrrespective of the length of post-lactational regression (Fig. 6). Similarly, *lactoferrin* and *κ-LC*, also demonstrated a parity-specific pattern of expression independent of length of regression (Fig. 6).

Parity-Related Molecular Markers are Conserved Across Species

Since our microarray analysis was conducted in mice, we were interested in determining if the parity-dependent differential expression of genes identified in this study were conserved among rodent species. We performed Northern analysis on pools of total RNA from 10 nulliparous and 10 parous Sprague-Dawley rats, a strain that has been widely used to study parity-induced protection against chemically-induced mammary tumorigenesis (Russo and Russo, 1978; Russo et al., 1979). Examination of the mammary epithelial cell differentiation markers, *β-casein, γ-casein, κ-casein*, and *lactoferrin*, confirmed that these genes are more highly expressed in the parous mammary gland (Fig. 7). Likewise, the immune cell markers *Ig-γ heavy chain, Igκ light chain*, and *MME* also display increased expression as a result of parity

(Fig. 7). In addition, many of the regulatory molecules that we have proposed to be involved in pathways functionally relevant to parity-induced protection, such as *AR* and *TGF-β3,* also demonstrate reproducible differential expression in the rat model. Of nine gene examined, we found all nine to be differentially expressed in the rat model in a manner similar to that observed in the mouse. This finding indicates that many of the parity-related molecular changes identified in this study are likely to be conserved among rodent species that display parity-induced protection against mammary tumorigenesis. Based on the functional and structural similarities between rodent and human mammary glands, we believe it probable that many of these molecular changes are also relevant in humans.

DISCUSSION

We have used DNA oligonucleotide microarrays to analyze the expression of ~11,000 genes and EST s in order to identify genes whose expression correlates with the protected state of the parous mammary gland. By our analysis, we have been able to detect both gross and subtle changes in gene expression that reproducibly distinguish between nulliparous and parous glands, as well as to identify changes in cell types that occur as a result of early parity. We have found molecular evidence that parity results in a more differentiated mammary epithelium, and that there is an increased contribution of the immune system in the parous gland. Finally, we have identified changes in regulatory pathways that have the potential to alter the susceptibility of the mammary epithelial cells to malignant transformation.

We proposed that a large fraction of the molecules isolated would serve to identify cell types within the mammary gland and would provide a means of describing the parous mammary

gland at a molecular level. By this analysis, we have isolated several genes whose expression corresponds to mammary epithelial cell differentiation. These genes include several milk protein genes as well as other genes whose expression is upregulated in terminally differentiated epithelium in preparation for lactation. Further analysis of several mammary epithelial cell differentiaton markers has indicated that these markers are useful in identifying populations of epithelial cell subtypes that have been expanded as a consequence of parity (D'Cruz, manuscript in preparation). If as our data suggests, expression of differentiation markers correlates with a more protected mammary gland, the expression of this subset of genes may provide clinically valuable information in terms of predicting breast cancer risk. Moreover, the relative distribution of cells marked by the expression of such genes may provide a molecular end point for targeted preventative strategies that attempt to differentiate breast epithelium.

Of note, the broad-based expression analysis of nulliparous and parous mammary glands described in this study also enabled us to distinguish changes in the cellular composition of the breast. We identified an abundance of genes that are preferentially expressed in the parous mammary gland and whose expression mark distinct classes of hematapoietic cells. We discovered that a permanent population of lymphocytes and macrophages resides within the parous mammary gland. The presence of these cells in the parous gland and the contribution of immune cells to providing parity-induced protection have not been previously addressed. One possible model for such a contribution includes heightened immunosurveillance in the parous gland. Although T-lymphocytes and natural killer cells are thought to mediate most anti-tumor immunity, antibody production against tumor antigens and selective lyses of tumor cells by macrophages also serve as anti-tumor mechanisms *in vivo* (Abbas et al., 1994). Consistent with

this model, we have identified the upregulation of *MHC Class I* in rats *and MHC Class II* in mice (data not shown) in parous epithelium, raising the possibility that antigen presentation to immune cells may also be enhanced as a result of parity.

The dramatic increase in epithelial expression of the cytokine Eta-*1* is suggestive of a model for paracrine signaling between epithelial and immune cells. Eta-1 binds to its cellular receptor, CD44, on the surface of several cell types, including mammary epithelial cells, macrophages, and fibroblasts (Naot et al., 1997). Signaling through this multifunctional receptor may enhance cell migration and the transmission of growth signals (Naot et al., 1997). Notably, Eta-1 expression has been documented to increase IgG and IgM expression in activated B cells, and may be partly responsible for the activity of B-lymphocytes within the parous breast (Weber and Cantor, 1996). In addition, it was recently reported that macrophages from mice deficient in *Eta-1* expression were found to produce decreased amounts of IL-12 and increased amounts of IL-10. Moreover, IFN-γ production was also decreased in these mice (Ashkar et al., 2000). Thus it appears that Eta-1 may influence cytokine production via interactions with macrophages and other immune cells. If in our system epithelial expression of *Eta-1* impacts the cytokine environment through macrophage stimulation, this may ultimately result in the activation of growth inhibitory pathways (Ashkar et al., 2000).

Growth promoting molecules represented the largest category of regulatory molecules identified by oligonucleotide microarray analysis. The reduction in expression of *AR, PTN, TSHR, Ob* and IGF-1, coupled with a decrease in expression of an EST with similarity to the growth hormone receptor, suggest that multiple mitogenic pathways may be downregulated in the more protected parous mammary gland. Interestingly, the elevated expression of *AR, PTN*

and *IGF-1* have been implicated in the progression of human breast cancer (Harris et al., 1992). The downregulation of *AR* and an EST that is homologous to the growth hormone receptor provide additional evidence for pathways that have previously been proposed to be altered as a consequence of parity (Thordarson et al., 1995). The identification of *IGF-1, PTN, Ob* and *TSHR*, however, are novel mitogenic pathways, whose association has not yet been correlated with reproductive history and breast cancer risk. The results of our studies, suggest that parity results in epithelium that has altered both systemic and local growth signaling pathways that ultimately contribute to a less mitogenic environment.

Finally, we have identified an increase in the expression of *TGF-β3* and its downstream transcriptional targets, *clusterin* and *Id-2*, in the parous mammary gland. The well-described functions of these molecules in growth inhibition implicate them as potential contributors to parity-induced protection. Moreover, exogenous expression of *TGF-β* in mammary epithelium is able to inhibit ductal morphogenesis and cause the regression of TEB, thus supporting this model. While our data do not conclusively demonstrate the activation of *TGF-β3* signaling in the parous breast, the coordinant regulation of downstream targets, such as *Id-2* and *clusterin* further support this claim. Other reported downstream targets such as *Bax*, *ICE* and *collagen III* did not demonstrate differential regulation by microarray analysis (Kretzschmar and Massague, 1998). In part, this may be due to tissue specificity of downstream targets, post-transcriptional regulation, or that the expression levels of these molecules are below the sensitivity of the technology. Therefore, investigation of the activation of additional molecules in the pathway, such as SMAD 2, SMAD 3 and SMAD 4 would provide additional insight into the activity of TGF-β3 signaling at the protein level. At present, the activation of a growth inhibitory pathway,

such as TGF-β3 presents a novel mechanism that could account for the decrease in susceptibility of the parous mammary gland to epithelial transformation.

We have isolated a comprehensive panel of genes whose expression correlates with the protective parous state following an early first full-term pregnancy. For a subset of these genes, we have investigated their parity-dependent expression following a longer period of involution. The expression of all the genes tested were insensitive to the length of regression following a pregnancy, suggesting that the differential patterns that we have identified are permanent. Moreover, the parity-specific pattern of expression of many of the genes discussed in this study have been demonstrated in the rat, and indicate that many of the molecular changes that we have defined are highly conserved and may be relevant to the human breast.

FIGURE LEGENDS

Figure 1. Nulliparous and parous mammary glands are readily distinguishable by gross morphology and share common features across rodent strains. Carmine-stained whole mounts from Sprague-Dawley rats and C57Bl/6 and FVB mice display increased ductal branching in the involuted parous mammary gland as compared to the age-matched nulliparous gland. Histological analysis of hematoxylin and eosin-stained sections demonstrates that the proportion of epithelial cells is equivalent in nulliparous and parous mammary glands.

Figure 2. Genes identified as differentially expressed by microarray analysis are confirmed by Northern hybridization. Probes were hybridized to Northern membranes containing three independent pools of total RNA from 15-20 age-matched nulliparous (lanes 1-3) and parous (lanes 4-6) animals. *SOD3, Ob, MUC18*, and an EST homologous to an Ig Super family protein were confirmed to be downregulated as a result of parity, while *κ-casein, ADFP, CEL, CA2,* and *ADA* were demonstrated to be upregulated. Equivalent epithelial cell content in nulliparous and parous animals is demonstrated by *CK18* expression. *β-actin* and 28S rRNA serve as controls for equal loading.

Figure 3. Markers for immune cells identify B-lymphocytes and macrophages in the parous mammary gland. (A) Northern analysis of three independent pools of mammary gland total RNA isolated from FVB mice demonstrates consistent increased expression of *MME, KLC* and *Eta-1* in the parous gland. *β-actin* is shown as a loading control. (B) Northern hybridization of the *MME, KLC* and *Eta-1* probe to total mammary gland RNA isolated at the indicated time

points is shown. Expression levels are compared to those of *β-actin* to account for dilutional effects due to larg-scale increases in milk protein gene expression during late pregnancy and lactation. (C) *In situ* hybridization analysis of *MME, KLC* and *Eta-1* expression at the indicated time points is shown. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with an ^{35}S-labeled *MME, KLC* and *Eta-1* specific antisense probes. No signal over background was detected in sections hybridized with a sense probes (data not shown). Exposure times were identical for all dark-field photomicrographs of each gene to illustrate changes in expression. Magnification 300x.

Figure 4. Epithelial expression of the growth factor amphiregulin is downregulated during pregnancy and remains lower in the involuted parous gland than in the age-matched nulliparous gland. (A) Northern analysis of three independent pools of mammary gland total RNA isolated from FVB mice demonstrates consistent decreased expression of *AR* in the parous gland. *β-actin* is shown as a loading control. (B) Northern hybridization of the *AR* probe to total mammary gland RNA isolated at the indicated time points is shown. Expression levels are compared to those of β-actin to account for dilutional effects due to larg-scale increases in milk protein gene expression during late pregnancy and lactation. The 28S rRNA band is shown as a control for equal loading. (C) *In situ* hybridization analysis of *AR* expression at the indicated time points is shown. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with an ^{35}S-labled *AR*-specific antisense probe. No signal over background was detected in sections hybridized with a sense *AR* probe. Exposure times were identical for all dark-field photomicrographs to illustrate changes in *AR* expression. Magnification 300x.

Figure 5. Increased *TGF-β3* and *clusterin* mRNA expression in the parous mammary gland are consistent with an increase in cell death regulatory pathways. (A) Northern analysis of three independent pools of mammary gland total RNA isolated from FVB mice demonstrates consistent increased expression of *TGF-β3* and *clusterin* in the parous gland. *β-actin* is shown as a loading control. (B) Northern hybridization of the *TGF-β3* and *clusterin* probe to total mammary gland RNA isolated at the indicated time points is shown. (C) *In situ* hybridization analysis of *TGF-β3* and *clusterin* expression at the indicated time points is shown. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with an ^{35}S-labeled *TGF-β3* and *clusterin* antisense probe. Magnification 300x.

Figure 6. Parity-Dependent Patterns of Expression are Independent of Length of Regression. Probes for *TGF-β3, clusterin, lactoferrin, Eta-1, κ-LC* and *β-actin* were hybridized to Northern membranes containing total RNA from pools of nulliparous and parous animals. The differential regulation of the genes identified remain consistent and are permananent throughout longer periods of involution.

Figure 7. The differential expression of genes identified in mice is conserved in rats. Probes specific to rat sequences for *AR, IgG, Kappa light chain (KLC), MME, TGF-β3, γ-Casein, β-casein, lactoferrin*, and *κ-casein* were hybridized to Northern membranes containing pools of total RNA from 10 parous and 10 nulliparous Sprague-Dawley rats. Both the direction and magnitude of change in expression between the nulliparous and parous states are comparable to that observed in mice.

Table 1: Differentially Regulated Molecular Markers

Genes Down-Regulated

Sequence Identity	Accession Number	Function	Avg. Fold Change	Northern
Amphiregulin	L41352	Growth Factor	2.8	Y
Pleiotrophin	D90225	Growth Factor	2	Y
EST-similar to Growth Hormone Receptor	AA066700	Growth Factor Receptor	19.4	
Insulin Like Growth Factor 1B-Precursor	W10072	Growth Factor	2.2	
EST-similar to Ig Super Family	AA059664		2.5	Y
Thyroid Stimulated Hormone Receptor	U02602	Hormone Receptor	2	Y
Obesity Protein	U18812	Cell Signalling	2	Y
MUC 18	AA088962	Cell Adhesion	2	Y
Superoxide Dismutase III (SOD3)	X84940	Oxidoreductase	2.5	Y
Vinculin	L18880	Cell Adhesion	4.1	
Fibronectin	M18194	Cell Adhesion	1.8	

Genes Up-Regulated

Sequence Identity	Accession Number	Function	Avg. Fold Change	Northern
Gamma-Casein	D10215	Milk	20	Y
Whey Acidic Protein	J00544	Milk	23.7	Y
Alpha-Casein	M36780	Milk	4.2	
Beta-Casein	X04490	Milk	3.3	Y
Kappa Casein	M10114	Milk	2.9	Y
Alpha-Lactalbumin	M80909	Milk	13	Y
LPS-Binding Protein	X99347	Milk	6.5	
Lysozyme P	M21050	Milk	2	
Lactoferrin	J03298	Iron Transport	4.8	Y
Carboxyl Ester Lipase	U37386	Lipid Degradation	6.6	Y
WDNM1	X93037	Protease Inhibitor	3.5	Y
Immunoglobulin M Heavy Chain	ET61785	Immunoglobulin Domain	30	Y
Immunoglobulin G Heavy Chain	ET61798	Immunoglobulin Domain	13.3	Y
Kappa Light Chain	X16678	Immunoglobulin Domain	7	Y
B cell receptor gene	L43568	Immunoglobulin Domain	8	
Hemopoietic-Specific Early Response Protein (A1)	L16462	Apoptosis	2	
ET-a1	X16151	Signal	8.8	Y
Clusterin	L08325	Apoptosis	2	Y
Transforming Growth Factor Beta-3	M32745	Apoptosis	1.9	Y
Serum Amyloid A Protein	M13521	Signal	2	
Butyrophilin	U67065	Glycoprotein	6.2	
Carbonic Anhydrase Isozyme II	K00811	Zinc	3	Y
Oct binding factor 1 (OBF-1)	U43788	Nuclear protein	3	
Transcription Regulatory Protein (A-myb)	L35261	Nuclear protein	2.1	
Id-2 Protein	M69293	Nuclear protein	2.3	Y
GLYCAM-1	M93428	Cell Adhesion	15	
CRBP1	X60367	Retinol-Binding	3.5	Y
Connexin 26	M81445	Gap Junction	3.1	Y
Connexin 30	Z70023	Gap Junction	7.6	
Folate-Binding Protein 1	ET63126	Receptor	2.6	Y
Adenosine Deaminase	M10319	Nucleotide Metabolism	3	Y
Adipocyte Differentiation Related Protein	M93275	Membrane	1.8	Y
Macrophage Metalloelastase	M82831	Metalloprotease	2.1	Y

Fig 1.

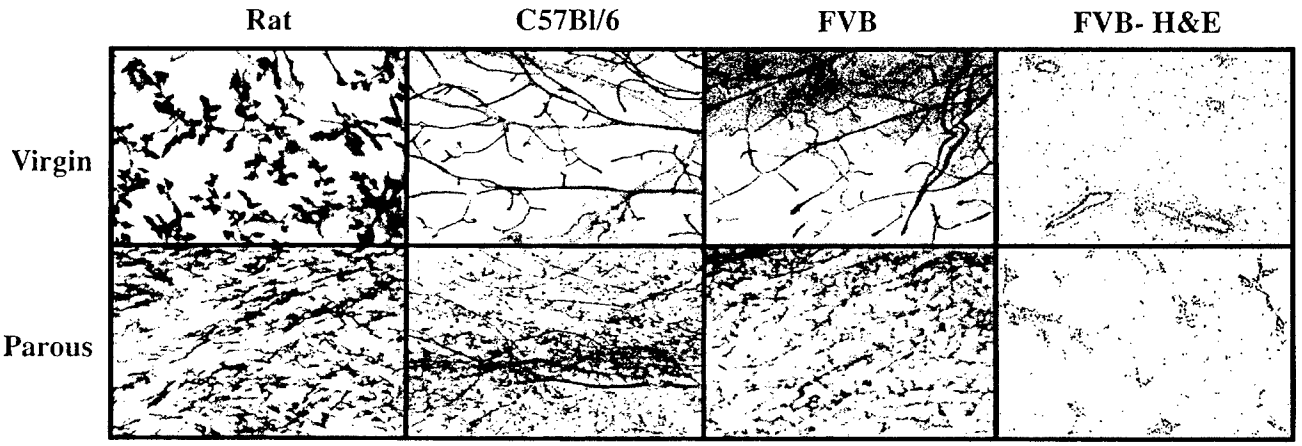


Fig. 2

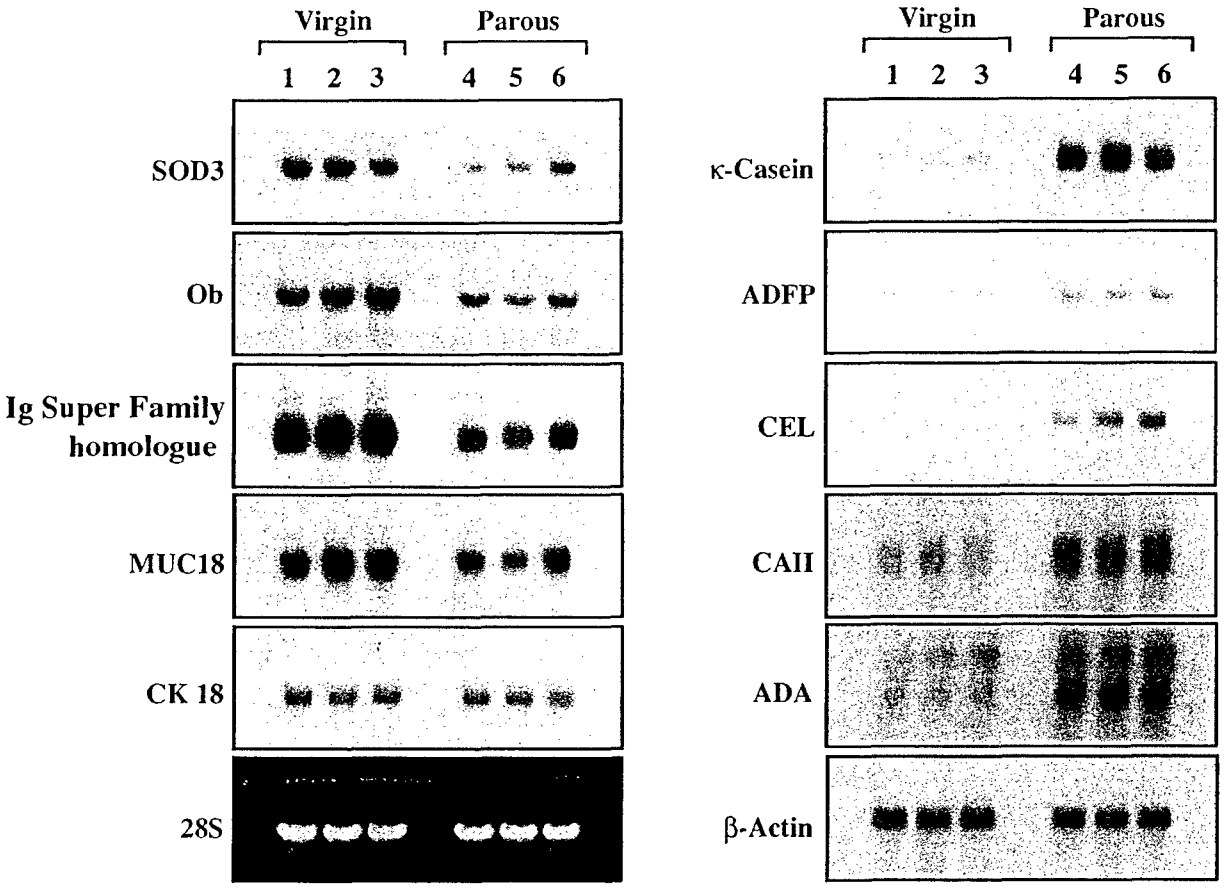

Virgin
Parous
1 2 3 4 5 6
SOD3
Ob
Ig Super Family homologue
MUC18
CK 18
28S
Virgin
Parous
1 2 3 4 5 6
κ-Casein
ADFP
CEL
CAII
ADA
β-Actin

Fig 3.

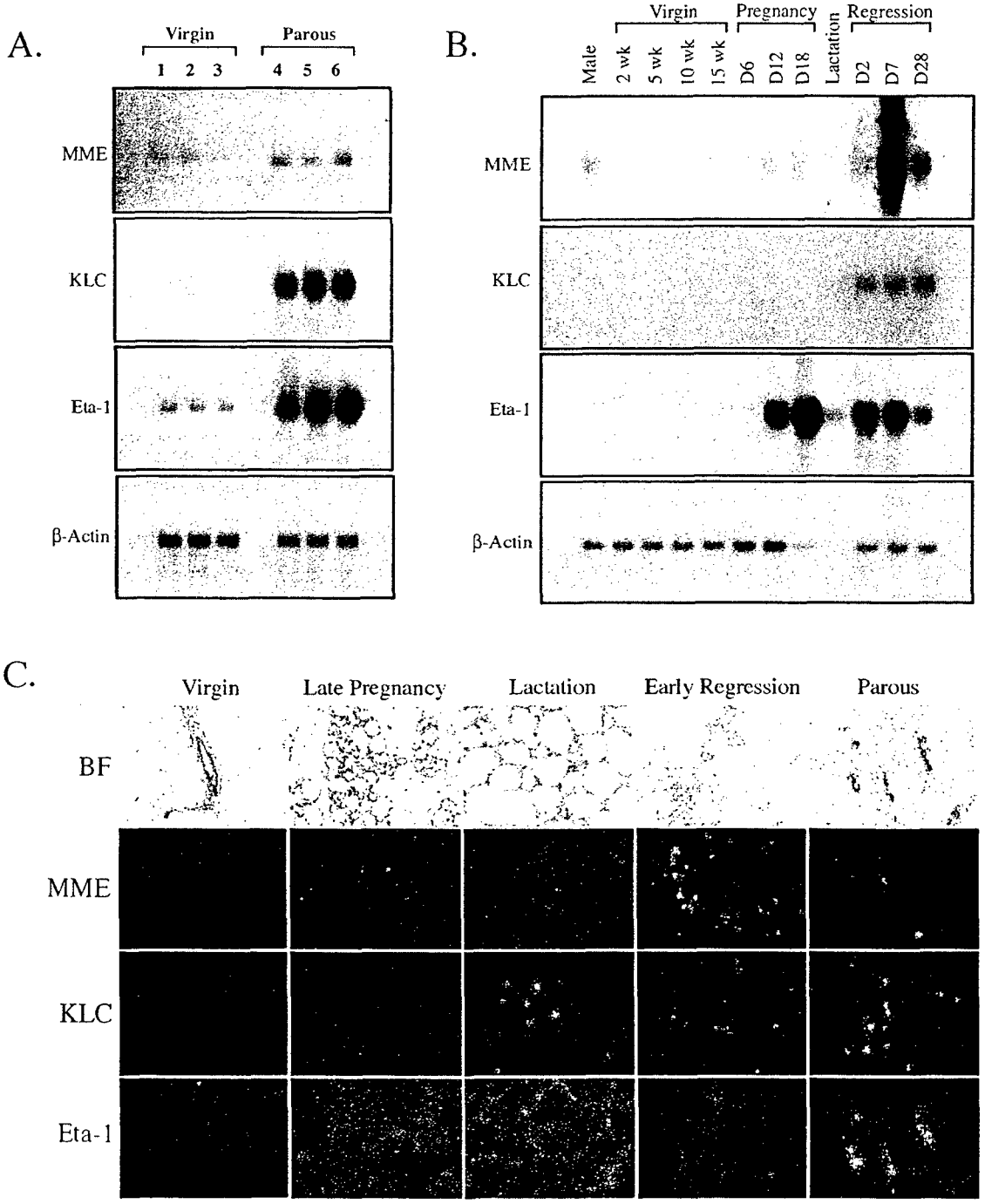
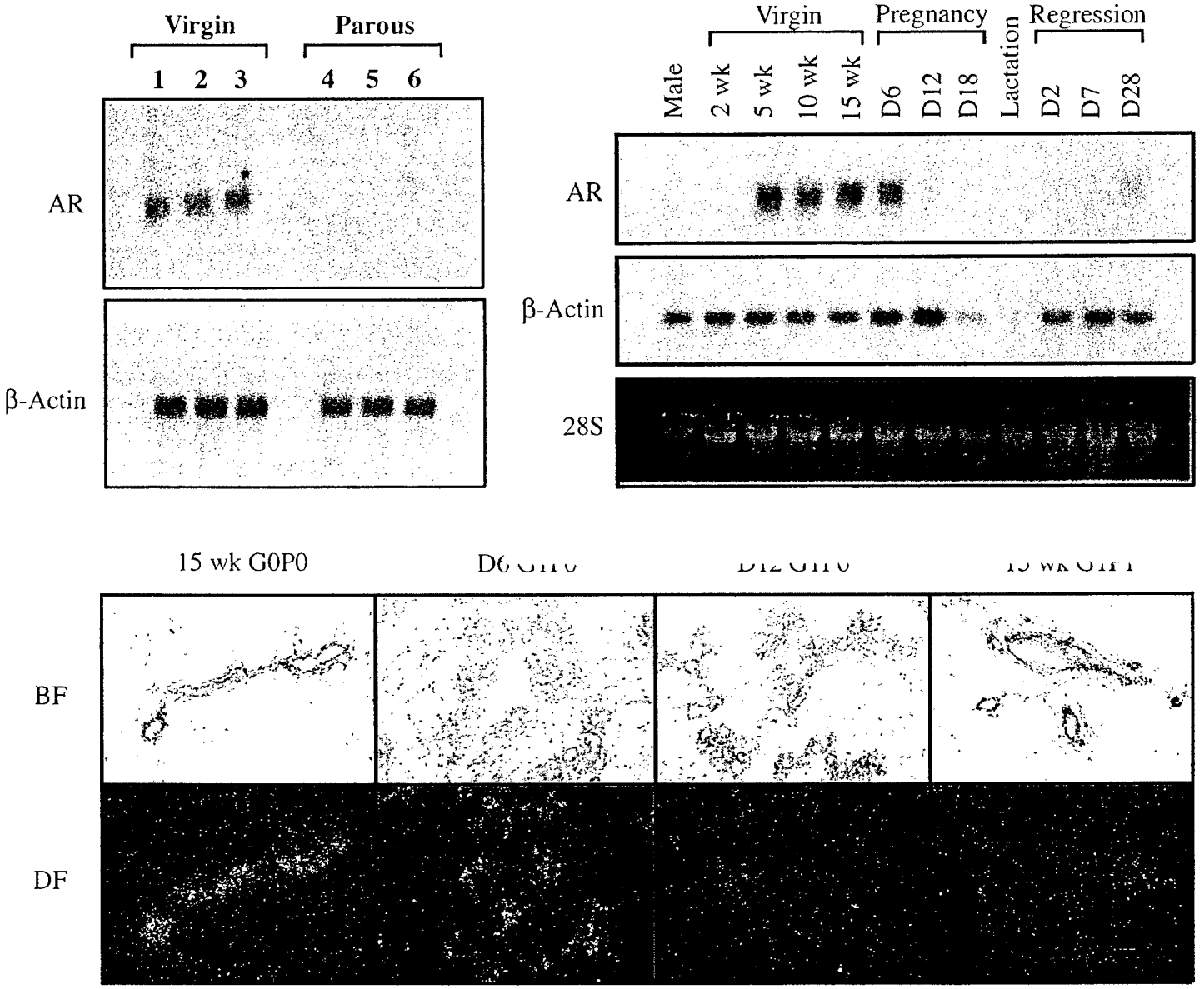

A.
Virgin
Parous
1 2 3
4 5 6
MME
KLC
Eta-1
β-Actin
B.
Virgin
Pregnancy
Regression
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
MME
KLC
Eta-1
β-Actin
C.
Virgin
Late Pregnancy
Lactation
Early Regression
Parous
BF
MME
KLC
Eta-1

Fig 4


Virgin
Parous
1 2 3
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Pregnancy
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2 wk
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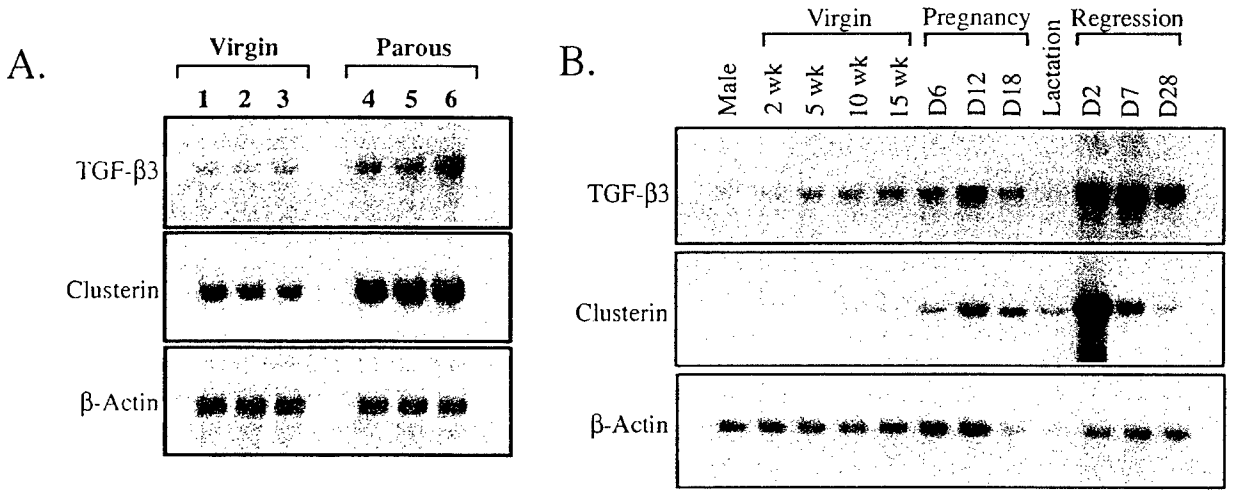
D12
D18
Lactation
D2
D7
D28
AR
β-Actin
28S
15 wk G0P0
BF
DF

Fig 5.


A.
Virgin
Parous
1 2 3
4 5 6
TGF-β3
Clusterin
β-Actin
B.
Virgin
Pregnancy
Regression
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
TGF-β3
Clusterin
β-Actin

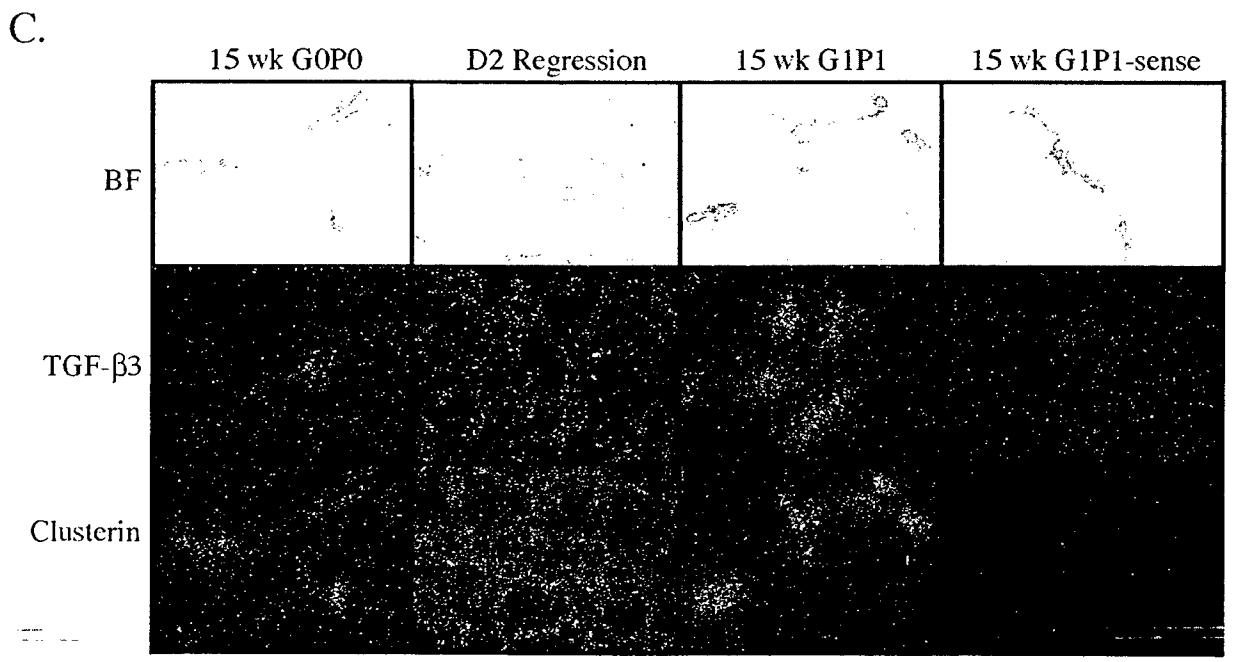

C.
15 wk G0P0
D2 Regression
15 wk G1P1
15 wk G1P1-sense
BF
TGF-β3
Clusterin

Fig 6.

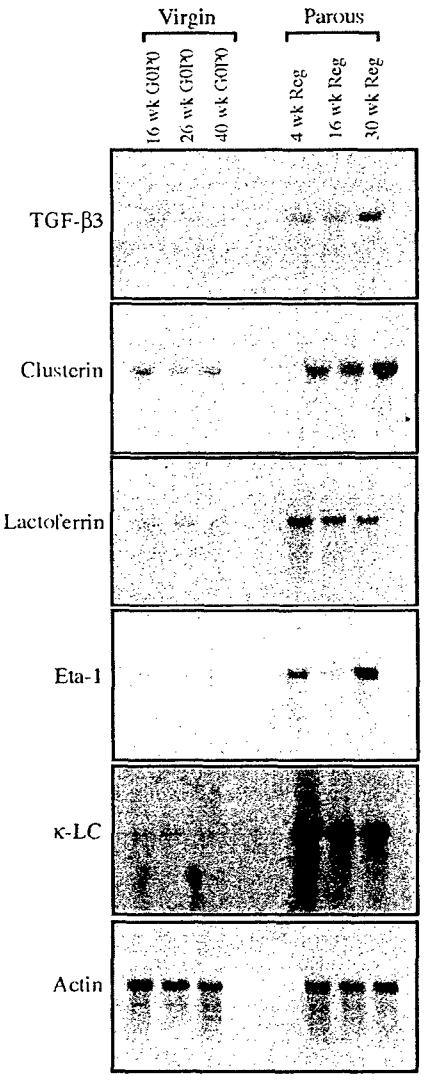
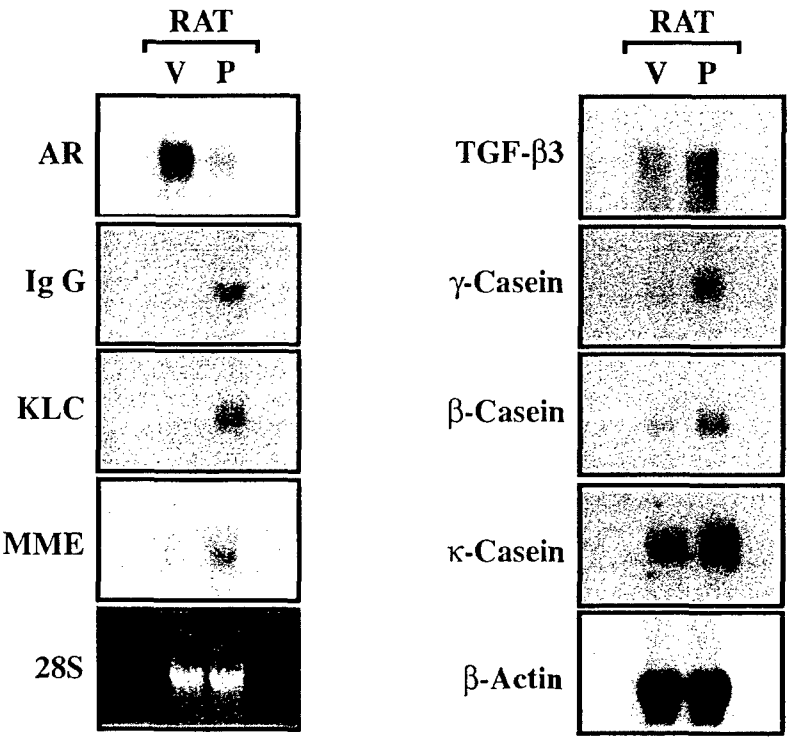

Virgin
Parous
16 wk G0P0
26 wk G0P0
40 wk G0P0
4 wk Reg
16 wk Reg
30 wk Reg
TGF-β3
Clusterin
Lactoferrin
Eta-1
κ-LC
Actin

Fig.7


RAT
V P
AR
Ig G
KLC
MME
28S
RAT
V P
TGF-β3
γ-Casein
β-Casein
κ-Casein
β-Actin

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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